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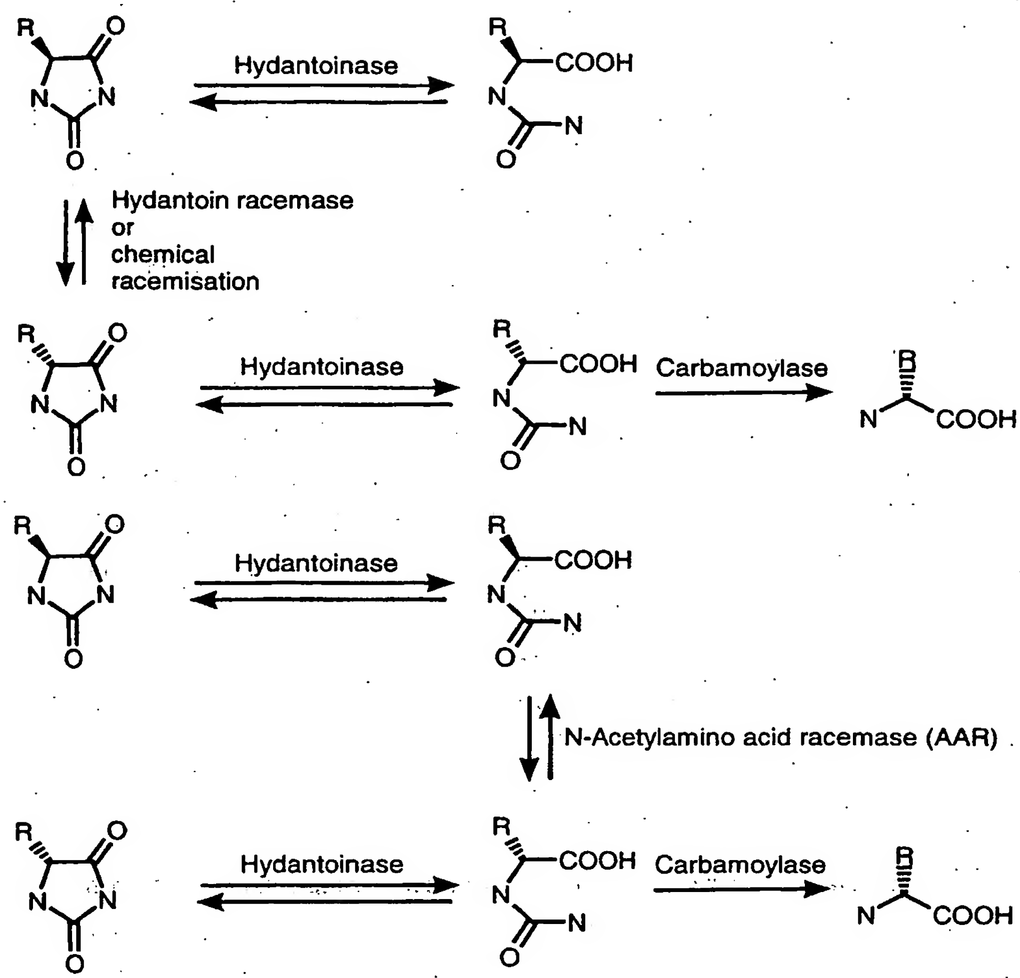
Screening process for hydantoin racemases

The present invention relates to a screening process for the detection of improved hydantoin racemases, to novel hydantoin racemases themselves and to their use in the preparation of N-carbamoyl-amino acids.

These optically active compounds are compounds that are frequently used in organic synthesis for the preparation of, for example, active ingredients having biological activity. They are also present in chiral auxiliaries, for example in the form of the amino alcohols (Evans reagents).

The enzymatic hydrolysis of 5-substituted hydantoins to N-carbamoyl-amino acids and the further reaction thereof to the corresponding enantiomerically enriched amino acids is a standard method in organic chemistry ("Enzyme Catalysis in Organic Synthesis", Eds.: Drauz, Waldmann, VCH, 1st and 2nd Ed.). The enantiodifferentiation can be carried out either at the stage of the hydantoin hydrolysis by means of hydantoinases or alternatively during the cleavage of the N-carbamoyl-amino acids by means of enantioselective carbamoylases. Because the enzymes each convert only one optical antipode of the corresponding compound, it is attempted to racemise the other in the mixture (*in situ*) in order to ensure the complete conversion of the hydantoin, which can readily be prepared racemically, into the corresponding enantiomerically enriched amino acid. The racemisation can proceed either at the stage of the hydantoins by means of chemical (base, acid, elevated temperature) or enzymatic processes or alternatively at the stage of the N-carbamoyl-amino acids by means of, for example, acetylamino acid racemases (DE10050124). By its nature, the latter variant is only successful if enantioselective carbamoylases are used. The following scheme illustrates this fact.

Scheme 1:



For aromatic substrates, the rate of the chemical racemisation of the hydantoins, as shown in Table 1, is sufficiently high to ensure high space-time yields for the preparation of amino acids by the hydantoinase process. For aliphatic hydantoins, such as isobutyl-, methyl- and isopropyl-hydantoin, however, the racemisation represents a considerable bottleneck in the synthesis of aliphatic amino acids.

Table 1: Racemisation constants of hydantoins at 40°C, pH 8.5 determined by initial rates according to a first-order reaction ($-k_{rac} = \ln([a]/[a_0])$ from: Hydrolysis and Formation of Hydantoins (Chpt. B 2.4). Syldatk, C. and Pietzsch, M. In: Enzyme catalysis in organic synthesis (Eds.: K. Drauz & H. Waldmann), VCH, 1st and 2nd Ed.).

5'-substituent	k_{rac} (h^{-1})	$t_{1/2}$ (h)
Phenyl	2.59	0.27
Methylthioethyl	0.12	5.82
Isobutyl	0.032	21.42
Methyl	0.02	33.98
Isopropyl	0.012	55.90

This problem manifests itself, for example, in the preparation, described in EP759475, of enantiomerically enriched tert.-butylhydantoin by means of the hydantoinase process. In this case, the complete conversion of 32 mM tert.-butylhydantoin with 1.5 kU R-hydantoinase required 8 days at pH 8.5 and 4 days at pH 9.5. The low space-time yield is in fact caused by the only slow chemical racemisation of tert.-butylhydantoin (k_{rac} = 0.009 h^{-1} at 50°C and pH 8.5).

There are known from the prior art hydantoin racemases from microorganisms of the genus *Pseudomonas, Microbacterium, Agrobacterium* and *Arthrobacter* (lit.: JP04271784; EP1188826; Cloning and characterization of genes from Agrobacterium sp. IP I-671 involved in hydantoin degradation. Hils, M.; Muench, P.; Altenbuchner, J.; Syldatk, C.; Mattes, R. Applied Microbiology and Biotechnology (2001), 57(5-6), 680-688; A new razemase for 5-monosubstituted hydantoins. Pietzsch, Markus;

Syldatk, Christoph; Wagner, Fritz. Ann. *N. Y. Acad. Sci. (1992), 672 (Enzyme Engineering XI), 478-83*. Lickefett, Holger; Krohn, Karsten; Koenig, Wilfried A.; Gehrcke, Barbel; Syldatk, Christoph. Tetrahedron: Asymmetry (1993), 4(6), 1129-35; Purification and characterization of the hydantoin razemase of Pseudomonas sp. strain NS671 expressed in Escherichia coli. Watabe, Ken; Ishikawa, Takahiro; Mukohara, Yukuo; Nakamura, Hiroaki. J. Bacteriol. (1992), 174(24), 7989-95).

Of the hydantoin racemases from *Arthrobacter aurescens* DSM 3745, *Pseudomonas sp.* NS671 and *Microbacterium liquefaciens*, it is known that these enzymes racemise aliphatic hydantoins, such as, for example, isopropylhydantoin or isobutylhydantoin, only weakly. It is also known that the hydantoin racemases from *Arthrobacter aurescens* DSM 3747 preferentially convert aromatic hydantoins, such as indolylmethylhydantoin or benzylhydantoin, whereas aliphatic hydantoins, such as methylthioethylhydantoin, are converted comparatively weakly or, in the case of isopropylhydantoin, are not converted at all (A new razemase for 5-monosubstituted hydantoins. Pietzsch, Markus; Syldatk, Christoph; Wagner, Fritz. Ann. N. Y. Acad. Sci. (1992), 672 (Enzyme Engineering XI), 478-83.).

The low activity of hydantoin racemases therefore frequently limits the economic potential of this route.

In order to enable as many hydantoin racemases as possible to be checked in a suitable time for their potential to racemise aliphatic hydantoins, the object of the present invention was *inter alia* to provide a suitable screening process for hydantoin racemases. Moreover, the screening process according to the invention should be usable as a component of a mutagenesis process for obtaining new and improved hydantoin racemases. It was also an object of the present invention to provide novel hydantoin racemases which are superior to the hydantoin racemases of the prior

art at least in terms of selectivity and/or activity and/or stability.

This object is achieved according to the claims. Claim 1 relates to a screening process for hydantoin racemases. Dependent claims 2 to 4 indicate advantageous embodiments of the screening process. Claim 5 is concerned with a mutagenesis process for the preparation of novel hydantoin racemases using the screening process according to the invention. Claims 6 to 11 relate to novel hydantoin racemases and to the nucleic acid sequences coding therefor and their use. Claims 12 to 14 are directed towards vehicles containing the hydantoin racemases according to the invention, or particular primers for their preparation.

By the provision of a screening process for hydantoin racemases, in which

a) an enantioselective hydantoinase and
b) the hydantoin racemase to be tested, which has a slower conversion rate compared with the hydantoinase under a), are allowed to act on
c) a chiral hydantoin, which is used in the opposite enantiomerically enriched form to the selectivity of the hydantoinase, and
d) the resulting N-carbamoyl-amino acid or the freed protons are detected in a time-dependent manner,

it becomes possible in a surprisingly simple and yet advantageous manner to check a large number of hydantoin racemases in a short time for their ability to racemise hydantoins in an improved manner.
By the use of an L-enantiomer of a 5'-monosubstituted hydantoin and the use of a D-selective hydantoinase which, on the basis of its enantioselectivity, preferably rapidly hydrolyses the resulting D-enantiomer of the hydantoin, the racemisation rate and hence the activity of the hydantoin racemase can be measured in a simple manner by the formation of the N-carbamoyl-D-amino acid or by freed

protons. The N-carbamoyl-amino acid can be quantified by methods known to the person skilled in the art, such as, for example, HPLC or colorimetric methods. Quantification *via* protons can be carried out in a simple manner *via* pH indicators, preferably cresol red. It should be noted that both D- and L-enantiomers of hydantoins having different optionally aliphatic 5'-substituents can be used in the process. When D-hydantoins are used, corresponding L-selective hydantoinases are to be used in the screening process.

In the process according to the invention there are advantageously used aliphatic hydantoins substituted in the 5'-position. In this context, aliphatically substituted hydantoins are understood to mean a system which has in the 5'-position on the hydantoin heterocycle a radical which is bonded to the heterocycle *via* a carbon atom having sp^3-hybridisation. Preferred 5'-substituents are methyl, ethyl, butyl, propyl, tertiary butyl, isopropyl and isobutyl. Ethylhydantoin is very particularly preferred.

There may be used as hydantoinases any hydantoinases known in the literature which enantioselectively hydrolyse the hydantoin enantiomer formed *via* the hydantoin racemase, it being necessary for this hydrolysis to be more rapid than the racemisation rate. Preferred hydantoinases are the commercial hydantoinases 1 & 2 from Roche, the hydantoinases of the genera *Agrobacterium, Arthrobacter, Bacillus, Pseudomonas, Flavobacterium, Pasteurella, Microbacterium, Vigna, Ochrobactrum, Methanococcus, Burkholderia* and *Streptomyces*.(Hils, M.; Muench, P.; Altenbuchner, J.; Syldatk, C.; Mattes, R. Cloning and characterization of genes from Agrobacterium sp. IP I-671 involved in hydantoin degradation. Applied Microbiology and Biotechnology (2001), 57(5-6), 680-688. Soong, C.-L.; Ogawa, J.; Shimizu, S. Cyclic ureide and imide metabolism in microorganisms producing a D-hydantoinase useful for D-

amino acid production. Journal of Molecular Catalysis B: Enzymatic (2001), 12(1-6), 61-70. Wiese, Anja; Wilms, Burkhard; Syldatk, Christoph; Mattes, Ralf; Altenbuchner, Josef. Cloning, nucleotide sequence and expression of a hydantoinase and carbamoylase gene from Arthrobacter aurescens DSM 3745 in Escherichia coli and comparison with the corresponding genes from Arthrobacter aurescens DSM 3747. Applied Microbiology and Biotechnology (2001), 55(6), 750-757. Yin, Bang-Ding; Chen, Yi-Chuan; Lin, Sung-Chyr; Hsu, Wen-Hwei. Production of D-amino acid precursors with permeabilized recombinant Escherichia coli with D-hydantoinase activity. Process Biochemistry (Oxford) (2000), 35(9), 915-921. Park, Joo-Ho; Kim, Geun-Joong; Lee, Seung-Goo; Lee, Dong-Cheol; Kim, Hak-Sung. Purification and characterization of thermostable D-hydantoinase from Bacillus thermocatenulatus GH-2. Applied Biochemistry and Biotechnology (1999), 81(1), 53-65; Pozo, C.; Rodelas, B.; de la Escalera, S.; Gonzalez-Lopez, J. D,L-Hydantoinase activity of an Ochrobactrum anthropi strain. Journal of Applied Microbiology (2002), 92(6), 1028-1034; Chung, Ji Hyung; Back, Jung Ho; Lim, Jae-Hwan; Park, Young In; Han, Ye Sun. Thermostable hydantoinase from a hyperthermophilic archaeon, Methanococcus jannaschii. Enzyme and Microbial Technology (2002), 30(7), 867-874; Xu, Zhen; Jiang, Weihong; Jiao, Ruishen; Yang, Yunliu. Cloning, sequencing and high expression in Escherichia coli of D-hydantoinase gene from Burkholderia pickettii. Shengwu Gongcheng Xuebao (2002), 18(2), 149-154; Las Heras-Vazquez, Francisco Javier; Martinez-Rodriguez, Sergio; Mingorance-Cazorla, Lydia; Clemente-Jimenez, Josefa Maria; Rodriguez-Vico, Felipe. Overexpression and characterization of hydantoin racemase from Agrobacterium tumefaciens C58. Biochemical and Biophysical Research Communications (2003), 303(2), 541-547; DE 3535987; EP 1275723; US 6087136; WO 0281626; US 2002045238; DE 4328829; WO 9400577; WO 9321336; JP

04325093; NL 9001680; JP 2003024074; WO 0272841; WO 0119982; WO 9620275).

The use of the hydantoinase from *Arthrobacter crystallopoietes*, especially from DSM 20117, is very particularly preferred.

As already indicated, the conversion rate of the hydantoinase should be superior to that of the racemase. The ratio of the rate constants of the hydantoinase to the hydantoin racemase (k_{hyd}/k_{rac}) is preferably > 2, particularly preferably > 10 and very particularly preferably > 50.

The invention also provides a process for the preparation of improved hydantoin racemases, which is distinguished by the fact that

a) the nucleic acid sequence coding for the hydantoin racemase is subjected to a mutagenesis,
b) the nucleic acid sequences obtainable from a) are cloned into a suitable vector and the vector is transferred into a suitable expression system, and
c) the resulting hydantoin racemases having improved activity and/or selectivity and/or stability are detected by means of a screening process according to the invention and isolated.

There may be used as starting genes for the mutagenesis of the hydantoin racemases any known hydantoin racemase genes mentioned in the listed literature. Preference is given to the hydantoin racemase genes of *Arthobacter, Pseudomonas, Agrobacterium* and *Micrococcus* (Wiese A; Pietzsch M; Syldatk C; Mattes R; Altenbuchner J Hydantoin racemase from Arthrobacter aurescens DSM 3747: heterologous expression, purification and characterization. JOURNAL OF BIOTECHNOLOGY (2000 Jul 14), 80(3), 217-30; Watabe K; Ishikawa T; Mukohara Y; Nakamura H Purification and characterization of the hydantoin racemase of Pseudomonas sp. strain NS671 expressed in Escherichia coli. JOURNAL OF

BACTERIOLOGY (1992 Dec), 174(24), 7989-95; Las Heras-Vazquez, Francisco Javier; Martinez-Rodriguez, Sergio; Mingorance-Cazorla, Lydia; Clemente-Jimenez, Josefa Maria; Rodriguez-Vico, Felipe. Overexpression and characterization of hydantoin racemase from Agrobacterium tumefaciens C58. Biochemical and Biophysical Research Communications (2003), 303(2), 541-547; EP 1188826). Very particular preference is given to the hydantoin racemase gene from *Arthrobacter aurescens,* which codes for the protein sequence in Seq.ID.No. 2.
For the mutagenesis of the hydantoin racemase there may be used any methods known in the literature, such as, for example, random mutagenesis, saturation mutagenesis, cassette mutagenesis or recombination methods (May, Oliver; Voigt, Christopher A.; Arnold, Frances H. Enzyme engineering by directed evolution. Enzyme Catalysis in Organic Synthesis (2nd Edition) (2002), 1 95-138; Bio/Technology 1991, 9, 1073-1077; Horwitz, M. and Loeb, L., Promoters Selected From Random DNA-Sequences, Proc Natl Acad Sci USA 83, 1986, 7405-7409; Dube, D. and L. Loeb, Mutants Generated By The Insertion Of Random Oligonucleotides Into The Active-Site Of The Beta-Lactamase Gene, Biochemistry 1989, 28, 5703-5707; Stemmer, P.C., Rapid evolution of a protein in vitro by DNA shuffling, Nature 1994, 370, 389-391 and Stemmer, P.C., DNA shuffling by random fragmentation and reassembly: In vitro recombination for molecular evolution. Proc Natl Acad Sci USA 91, 1994, 10747-10751).
The cloning and expression can be carried out as in the literature mentioned hereinbelow. The process can be carried out several times in succession, optionally with varying mutagenesis strategies.

The invention also provides rec-polypeptides or the nucleic acid sequences coding therefor, which are obtainable by the mutagenesis process mentioned above.

Another aspect of the invention is the use of the polypeptides so prepared in the preparation of chiral enantiomerically enriched N-carbamoyl-amino acids or amino acids. The nucleic acid sequences prepared according to the invention can be used in the preparation of whole cell catalysts.

Hydantoin racemases which have in position 79 an amino acid substitution with an amino acid selected from the group consisting of A, R, N, D, C, Q, E, H, I, L, K, M, F, P, S, T, Y and V also form part of the present invention. It is interesting that the amino acids surrounding this position are retained completely for many hydantoin racemases. The consensus sequence reads: FX_1DX_2GL (Seq.ID.No. 1), wherein X_2 represents P or T and X_1 represents W or G. Preferred mutants therefore contain the above-mentioned consensus sequence, X_1 preferably representing an amino acid selected from the group consisting of A, R, N, D, C, Q, E, H, I, L, K, M, F, P, S, T, Y and V. X_1 corresponds to position 79. Preferred mutants are shown in Table 2.

Table 2:

Mutant name	Mutation (codon)	Mutation X_1 (amino acid)	Activity change	Seq. ID No.
3CH11	GGG -> GAG	G79E	2	5
1BG7	GGG -> AGG	G79R	2	3
BB5	GGG -> TTG	G79L	4	9
AE3	GGG -> CAG	G79Q	4	7

Further extremely advantageous combinations of X_1 and X_2 hydantoin racemases are listed in Table 3 below.

Table 3: Advantageous combinations of X_1 and X_2 in the consensus motif FX_1DX_2GL

X_1	L	E	Q	R	L	E	Q	R
X_2	P	P	P	P	T	T	T	T

It is particularly advantageous if the hydantoin racemases contain the above-mentioned consensus region and additionally exhibit a homology of >40% with the hydantoin racemase from DSM 20117.

The invention also provides isolated nucleic acid sequences coding for a hydantoin racemase selected from the group:

a) a nucleic acid sequence coding for a hydantoin racemase according to the invention,
b) a nucleic acid sequence which hybridises under stringent conditions with the nucleic acid sequence coding for a hydantoin racemase according to the invention or with the sequence complementary thereto,
c) a nucleic acid sequence according to Seq.ID.No. 3, 5, 7 or 9 or a nucleic acid sequence having a homology of > 80% therewith,
d) a nucleic acid sequence containing 15 successive nucleotides of sequences Seq.ID.No. 3, 5, 7 or 9.

With regard to point d), it is preferred for the nucleotide sequence according to the invention to contain 20, more preferably 25, yet more preferably 30, 31, 32, 33, 34 and most preferably more than 34 identical consecutive nucleic acids of the sequences Seq.ID.No. 3, 5, 7 or 9.

As mentioned, the invention also includes nucleic acid sequences which hybridise under stringent conditions with the single-strand nucleic acid sequences according to the invention or with their complementary single-strand nucleic acid sequences (b), or nucleic acid sequences which are alike in sequence sections (d). Particular gene probes or

the primers necessary for a PCR, for example, are to be regarded as such.

Coupling of hydantoin racemase and hydantoinase and optionally carbamoylase can be carried out by bringing together the free or immobilised enzymes. However, it is preferred for the hydantoinase to be expressed in the same cell together with the hydantoin racemase and/or the carbamoylase (whole cell catalyst).
The nucleic acid sequences according to the invention can therefore be cloned into a whole cell catalyst as a constituent of a gene in a manner analogous to that in DE10234764 and the literature cited therein.
Provided that the latter then also contains genes for a hydantoinase and/or carbamoylase, it is capable of converting racemic hydantoins completely into enantiomerically enriched amino acids. Without a cloned carbamoylase gene, the reaction stops at the stage of the N-carbamoyl-amino acids.
The host organism used is preferably an organism as mentioned in DE10155928. The advantage of such an organism is the simultaneous expression of all the enzymes involved, with which only a rec-organism must be used for the total reaction.
In order to match the expression of the enzymes in respect of their conversion rates, the corresponding coding nucleic acid sequences can be cloned into different plasmids with different copy numbers and/or promoters of different strengths can be used for a different strength of expression of the nucleic acid sequences. In such matched enzyme systems, there is advantageously no accumulation of an intermediate compound which may have an inhibiting action, and the reaction under consideration can proceed at an optimum overall rate. This is sufficiently well known to the person skilled in the art, however (Gellissen, G.; Piontek, M.; Dahlems, U.; Jenzelewski, V.; Gavagan, J. W.; DiCosimo, R.; Anton, D. L.; Janowicz, Z. A. (1996),

Recombinant Hansenula polymorpha as a biocatalyst. Coexpression of the spinach glycolate oxidase (GO) and the S. cerevisiae catalase T (CTT1) gene, Appl. Microbiol. Biotechnol. 46, 46-54; Farwick, M.; London, M.; Dohmen, J.; Dahlems, U.; Gellissen, G.; Strasser, A. W.; DE19920712). The preparation of such a whole cell catalyst is sufficiently well known to the person skilled in the art (Sambrook, J.; Fritsch, E. F. and Maniatis, T. (1989), Molecular cloning: a laboratory manual, 2nd ed., Cold Spring Harbor Laboratory Press, New York; Balbas, P. and Bolivar, F. (1990), Design and construction of expression plasmid vectors in E. coli, Methods Enzymol. 185, 14-37; Rodriguez, R.L. and Denhardt, D. T (eds) (1988), Vectors: a survey of molecular cloning vectors and their uses, 205-225, Butterworth, Stoneham).

In a next embodiment, the invention relates to plasmids or vectors containing one or more of the nucleic acid sequences according to the invention.
Suitable plasmids or vectors are in principle any forms available to the person skilled in the art for this purpose. Such plasmids and vectors will be found, for example, in Studier *et al.* (Studier, W. F.; Rosenberg A. H.; Dunn J. J.; Dubendroff J. W.; (1990), Use of the T7 RNA polymerase to direct expression of cloned genes, Methods Enzymol. 185, 61-89) or the brochures of Novagen, Promega, New England Biolabs, Clontech or Gibco BRL. Further preferred plasmids and vectors can be found in: Glover, D. M. (1985), DNA cloning: a practical approach, Vol. I-III, IRL Press Ltd., Oxford; Rodriguez, R.L. and Denhardt, D. T (eds) (1988), Vectors: a survey of molecular cloning vectors and their uses, 179-204, Butterworth, Stoneham; Goeddel, D. V. (1990), Systems for heterologous gene expression, Methods Enzymol. 185, 3-7; Sambrook, J.; Fritsch, E. F. and Maniatis, T. (1989), Molecular cloning: a laboratory manual, 2nd ed., Cold Spring Harbor Laboratory Press, New York.

Plasmids with which the gene construct containing the nucleic acid according to the invention can be cloned into the host organism in a very preferred manner are derivatives of pUC18 and pUC19 (Roche Biochemicals), pKK-177-3H (Roche Biochemicals), pBTac2 (Roche Biochemicals), pKK223-3 (Amersham Pharmacia Biotech), pKK-233-3 (Stratagene) or pET (Novagen). Further preferred plasmids are pBR322 (DSM3879), pACYC184 (DSM4439) and pSC101 (DSM6202), which can be obtained from DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany.

The invention is likewise directed towards microorganisms containing one or more nucleic acid sequences according to the invention. The microorganism into which the plasmids containing the nucleic acid sequences according to the invention are cloned serves to multiply and obtain a sufficient amount of the recombinant enzyme. The processes therefor are well known to the person skilled in the art (Sambrook, J.; Fritsch, E. F. and Maniatis, T. (1989), Molecular cloning: a laboratory manual, 2nd ed., Cold Spring Harbor Laboratory Press, New York). In principle, there can be used as microorganisms any organisms suitable to the person skilled in the art for this purpose, such as, for example, yeasts, such as *Hansenula polymorpha*, *Pichia sp.*, *Saccharomyces cerevisiae*, prokaryotes, such as *E. coli*, *Bacillus subtilis*, or eukaryotes, such as mammalian cells, insect cells. *E. coli* strains are preferably to be used for this purpose. Very particular preference is given to: *E. coli* XL1 Blue, W3110, DSM14459 (PCT/US00/08159), NM 522, JM101, JM109, JM105, RR1, DH5α, TOP 10⁻ and HB101. Plasmids with which the gene construct containing the nucleic acid according to the invention is preferably cloned into the host organism are indicated above.

A following aspect of the invention is directed towards primers for the preparation of the gene sequences according to the invention by means of any type of PCR. Included are

the sense and antisense primers coding for the corresponding amino acid sequences, or complementary DNA sequences. Suitable primers can in principle be obtained by processes known to the person skilled in the art. The location of the primers according to the invention is carried out by comparison with known DNA sequences or by translation of the amino acid sequences under consideration into the preferred codon of the organism in question (e.g. for Streptomyces: Wright F. and Bibb M. J. (1992), Codon usage in the G+C-rich *Streptomyces* genome, Gene 113, 55-65). Similarities in the amino acid sequence of proteins of so-called superfamilies are likewise of use therefor (Firestine, S. M.; Nixon, A. E.; Benkovic, S. J. (1996), Threading your way to protein function, Chem. Biol. 3, 779-783). Further information hereon can be found in Gait, M. J. (1984), Oligonucleotide synthesis: a practical approach, IRL Press Ltd., Oxford; Innis, M. A.; Gelfound, D. H.; Sninsky, J. J. and White, T.J. (1990), PCR Protocols: A guide to methods and applications, Academic Press Inc., San Diego.
Preferred primers are those of Seq.ID.No. 11 and 12.

As already indicated, the enzymes under consideration (hydantoin racemase, hydantoinases and/or carbamoylases) can be used in free form as homogeneously purified compounds or as an enzyme prepared by recombinant methods (rec-). The enzymes may also be used as a constituent of an intact guest organism or in conjunction with the cell mass of the host organism which has been opened up and highly purified as desired.
It is also possible to use the enzymes in immobilised form (Sharma B. P.; Bailey L. F. and Messing R. A. (1982), Immobilisierte Biomaterialiern - Techniken und Anwendungen, Angew. Chem. 94, 836-852). Immobilisation is preferably carried out by lyophilisation (Paradkar, V. M.; Dordick, J. S. (1994), Aqueous-Like Activity of α-Chymotrypsin Dissolved in Nearly Anhydrous Organic Solvents, J. Am.

Chem. Soc. 116, 5009-5010; Mori, T.; Okahata, Y. (1997), A variety of lipi-coated glycoside hydrolases as effective glycosyl transfer catalysts in homogeneous organic solvents, Tetrahedron Lett. 38, 1971-1974; Otamiri, M.; Adlercreutz, P.; Matthiasson, B. (1992), Complex formation between chymotrypsin and ethyl cellulose as a means to solbilize the enzyme in active form in toluene, Biocatalysis 6, 291-305). Very special preference is given to lyophilisation in the presence of surface-active substances, such as Aerosol OT or polyvinylpyrrolidone or polyethylene glycol (PEG) or Brij 52 (diethylene glycol monocetyl ether) (Kamiya, N.; Okazaki, S.-Y.; Goto, M. (1997), Surfactant-horseradish peroxidase complex catalytically active in anhydrous benzene, Biotechnol. Tech. 11, 375-378).
Very special preference is given to immobilisation on Eupergit®, especially Eupergit C® and Eupergit 250L® (Röhm) (Eupergit.RTM. C, a carrier for immobilization of enzymes of industrial potential. Katchalski-Katzir, E.; Kraemer, D. M. Journal of Molecular Catalysis B: Enzymatic (2000), 10(1-3), 157-176.)

Also preferred is immobilisation on Ni-NTA in combination with the polypeptide supplemented with the His tag (hexa-histidine) (Purification of proteins using polyhistidine affinity tags. Bornhorst, Joshua A.; Falke, Joseph J. Methods in Enzymology (2000), 326, 245-254). Use as CLECs is also conceivable (St. Clair, N.; Wang, Y.-F.; Margolin, A. L. (2000), Cofactor-bound cross-linked enzyme crystals (CLEC) of alcohol dehydrogenase, Angew. Chem. Int. Ed. 39, 380-383).
By means of these measures it can be possible to generate from polypeptides that are rendered unstable by organic solvents polypeptides that are stable and can work in mixtures of aqueous and organic solvents or in wholly organic solvents.

Whole cell catalysts are generally used in the form of free or immobilised cells. For this purpose, the active cell mass is re-suspended in a hydantoin-containing solution. The cell concentration is from 1 to 100 g/l. The concentration of hydantoin is from 0.1 to 2 molar. H_2O is preferably used as the solvent, but mixtures of organic solvents and H_2O can also be used. The pH value is either not controlled or is maintained between pH 6 and pH 10 by means of conventional buffers or by continuous pH monitoring. The reaction temperature is typically from 20°C to 90°C. In dependence on the hydantoinase used, divalent metal ions are added in concentrations of from 0.1 to 5 mM. Preferred metal ions are Mn^{2+}, Zn^{2+} or Co^{2+}.
With regard to the use of the individual enzymes, an equivalent procedure can be employed.

The products prepared by the use of the hydantoin racemases according to the invention in the manner as described, for example, above are worked up by conventional methods. However, working up by ion exchange chromatography is preferred. As a result, the product is freed of the salts formed in the reaction. The eluate is optionally clarified using activated carbon and the resulting enantiomerically enriched amino acid or N-carbamoyl-amino acid is precipitated by concentration of the solvent and dried.

Coupling of an enzymatic racemisation with an enantioselective hydrolysis for the screening of hydantoin racemase activities has not hitherto been used to produce improved hydantoin racemases. For the process according to the invention to be applied particularly successfully, several requirements should be met:

1. The chemical racemisation rate of the enantiomerically pure hydantoin used in the screeing must be very much lower than the rate of the enzymatically catalysed reaction.

2. The enantioselective hydrolysis by means of the hydantoinase must take place very much more rapidly than the enzymatic racemisation of the hydantoin.

For aliphatically substituted hydantoins, point 1 is met owing to their slow chemical racemisation. Point 2 can be fulfilled by a targeted selection of suitable hydantoinases (see hereinabove).

The present invention is not rendered obvious by the statements made in the prior art, because no indications are to be found therein relating to the requirements mentioned hereinbefore.

All the indicated mutants exhibit a mutation at amino acid position 79, which for the first time indicates the importance of this position for the enzyme function. It is interesting that the amino acids surrounding this position are retained completely for all known hydantoin racemases. This shows that, for other hydantoin racemases which contain the above-described sequence motif and exhibit a high degree of homology (>40% sequence identity), improved enzyme variants can be produced by site-specific mutagenesis at position 79, which could not hitherto be derived from the prior art.

Within the scope of the invention, the expression optically enriched (enantiomerically enriched) compounds is understood to mean the presence of one optical antipode in admixture with the other in >50 mol.%.

The expression nucleic acid sequences includes all types of single-strand or double-strand DNA as well as RNA or mixtures thereof.

According to the invention, the improvement in the activity and/or selectivity and/or stability means that the polypeptides are more active and/or more selective or less selective or more stable under the reaction conditions.

While the activity and the stability of the enzymes should naturally be as high as possible for technical application, the selectivity is said to be improved when either the substrate selectivity falls but the enantioselectivity of the enzymes is increased.

According to the invention, the claimed polypeptides and the nucleic acid sequences also include those sequences which exhibit a homology (excluding natural degeneration) of greater than 70% (in respect of the nucleic acid sequence) or > 40% or 80% (in respect of the polypeptides), preferably greater than 90%, 91%, 92%, 93% or 94%, more preferably greater than 95% or 96% and particularly preferably greater than 97%, 98% or 99%, with one of these sequences, provided that the mode of action or purpose of such a sequence is retained. The expression "homology" (or identity) as used herein can be defined by the equation $H (\%) = [1 - V/X] \times 100$, where H means homology, X is the total number of nucleobases/amino acids in the comparison sequence and V is the number of different nucleobases/amino acids of the sequence under consideration relative to the comparison sequence. In any case, the expression nucleic acid sequences coding for polypeptides includes all sequences that appear possible according to the degeneration of the genetic code.

The expression "under stringent conditions" is understood herein as described in Sambrook *et al.* (Sambrook, J.; Fritsch, E. F. and Maniatis, T. (1989), Molecular cloning: a laboratory manual, 2nd ed., Cold Spring Harbor Laboratory Press, New York). A stringent hybridisation according to the present invention is preferably present when, after washing for one hour with 1 x SSC (150 mM sodium chloride, 15 mM sodium citrate, pH 7.0) and 0.1 % SDS (sodium dodecylsulfate) at 50°C, preferably at 55°C, more preferably at 62°C and most preferably at 68°C, and more preferably for 1 hour with 0.2 x SSC and 0.1 % SDS at 50°C, more preferably

at 55°C, yet more preferably at 62°C and most preferably at 68°C, a positive hybridisation signal is still observed.

The literature references cited in this specification are incorporated in the disclosure by reference.

The organism *Arthrobacter aurescens* DSM3747 was deposited with Deutsche Sammlung für Mikroorganismen GmbH, Mascheroder Weg 1b, 38124 Braunschweig by Rütgerswerke Aktiengesellschaft on 28.05.86.

Examples

Example 1: Production of hydantoin racemase mutants - random mutagenesis

0.25 ng of the vector pOM21 (plasmid map see Fig.1; sequence see Seq.ID.No.13) (PCT/US00/08159) was used as template in a 100 µl PCR reaction mix consisting of PCR buffer (10 mM Tris, 1.5 mM MgCl2, 50 mM KCl, pH 8.5), 200 µM dTTP, 200 µM dGTP, 200 µM dATP, 200 µM dCTP, 50 pmol. of the respective primer (see Seq.ID.No.11 and 12) and 2.5 U Taq polymerase (Roche). After 30 cycles, the amplified product was purified by means of gel extraction (QiaexII gel extraction kit) and subcloned into the vector pOM21 by means of the restriction enzymes NdeI and PstI. The ligation product was used for the transformation of hydantoinase-positive strains (see Example 2).

Example 2: Preparation of hydantoinase-positive strains and of a mutant library

Chemically competent *E. coli* JM109 (e.g. from Promega) were transformed with 10 ng of the plasmid pDHYD (see Fig.2; see Seq.ID.No. 15), which carries the D-hydantoinase gene from *Arthrobacter crystallopoietes* DSM20117 under the control of a rhamnose promoter. The complete sequence of the plasmid is shown in Seq.ID.No. 15. The hydantoinase-positive strain so produced was in turn rendered chemically competent and transformed for the preparation of the mutant library with the ligation product of the hydantoin racemase random mutagenesis from Example 1. The colonies of the mutant library were spread onto ampicillin- and chloramphenicol-containing agar plates and then subjected to a screening, which is described in Example 3.

Example 3: Screening for hydantoin racemase mutants having improved enzyme properties

Individual colonies of the mutant library were inoculated in 96-well plates which were filled with 100 µl per well of LB medium (5 g/l yeast extract, 10 g/l trypton, 10 g/l NaCl) supplemented with rhamnose (2 g/l) and $ZnCl_2$ (1 mM). The plates were incubated for 20 hours at 30°C. 100 µl of screening substrate (100 mM L-ethylhydantoin, 50 mg/l cresol red, pH 8.5) were then added to each well and the plates were incubated for 4 hours at 20°C. Wells having improved hydantoin racemase mutants could be identified directly with the eye by means of a more intense yellow colouration compared with the wild type, or using a spectral photometer at 580 nm.

Example 4: Characterisation of hydantoin racemase mutants having improved enzyme properties

The racemase mutants identified in the screening were subsequently tested by means of HPLC analysis for their activity in comparison with the wild type, and the corresponding mutations were determined by means of sequencing. For this purpose, plasmids were isolated from individual colonies of the different clones (Qiagen Mini-Prep Kit) and sequenced. The same clones were used to produce active biomass. An overnight culture (OD_{600}=4) of the respective clones was to this end diluted 1:100 in 100 ml of LB medium (5 g/l yeast extract, 10 g/l trypton, 10 g/l NaCl) supplemented with rhamnose (2 g/l) and $ZnCl_2$ (1 mM) and incubated for 18 hours at 30°C and 250 rpm. The biomass was pelletised by centrifugation (10 min, 10,000 g) and the supernatant was discarded. 2 g of active biomass were then re-suspended in 50 ml of the substrate solution (100 mM L-ethylhydantoin, pH 8.5) and incubated at 37°C. Samples were taken after various times, the biomass was separated off by centrifugation (5 min, 13,000 rpm) and the

supernatant was analysed by means of HPLC for the concentration of the N-carbamoyl-aminobutyric acid formed.

Example 5: Preparation of L-amino acids using improved hydantoin racemases

A strain of *E. coli* JM109 transformed with pOM21-BB5 and pOM22 Fig. 3 (see Seq.ID.No.14) (PCT/US00/08159) was incubated at 30°C for 18 hours, with shaking (250 rpm), in LB medium which contained ampicillin (100 µg/l) and chloramphenicol (50 µg/l) and to which 2 g/l of rhamnose had been added. The biomass was pelletised by centrifugation and re-suspended in a corresponding volume of 100 mM DL-ethylhydantoin solution, pH 8.5, and 1 mM $CoCl_2$, so that a cell concentration of 30 g/l was obtained. This reaction solution was incubated for 10 hours at 37°C. The cells were then separated off by centrifugation (30 min, 5000 g) and the clear supernatant was analysed by means of HPLC for the resulting amino acid. For working up the resulting amino acid, the volume of the supernatant was reduced to half, and methanol was added 1:2. The precipitated amino acid was then filtered off and dried. The total yield of the amino acid was >60%.

Example 6: Preparation of D-amino acids using improved hydantoin racemases

A strain of *E. coli* JM109 transformed with pOM21-BB5 and pJAVIER16 Fig. 4 (see Seq.ID.No.16) was incubated at 30°C for 18 hours, with shaking (250 rpm), in LB medium which contained ampicillin (100 µg/l) and chloramphenicol (50 µg/l) and to which 2 g/l of rhamnose had been added. The biomass was pelletised by centrifugation and re-suspended in a corresponding volume of 100 mM DL-ethylhydantoin solution, pH 8.5, and 1 mM $CoCl_2$, so that a cell concentration of 30 g/l was obtained. This reaction solution was incubated for 10 hours at 37°C. The cells were then separated off by centrifugation (30 min, 5000 g) and

the clear supernatant was analysed by means of HPLC for the resulting amino acid. For working up the resulting amino acid, the volume of the supernatant was reduced to half, and methanol was added 1:2. The precipitated amino acid was then filtered off and dried. The total yield of the amino acid was >60%.

Patent claims:

1. Screening process for hydantoin racemases, characterised in that
 a) an enantioselective hydantoinase and
 b) the hydantoin racemase to be tested, which has a slower conversion rate compared with the hydantoinase under a), are allowed to act on
 c) a chiral hydantoin, which is used in the opposite enantiomerically enriched form to the selectivity of the hydantoinase, and
 d) the resulting N-carbamoyl-amino acid or the freed protons are detected in a time-dependent manner.

2. Process according to claim 1, characterised in that an aliphatically substituted hydantoin is used.

3. Process according to one or more of the preceding claims, characterised in that a hydantoinase from *Arthrobacter crystallopoietes* is used.

4. Process according to one or more of the preceding claims, characterised in that the ratio of the rate constants of the hydantoinase to the hydantoin racemase (k_{hyd}/k_{rac}) is > 2.

5. Process for the preparation of improved hydantoin racemases, characterised in that
 a) the nucleic acid sequence coding for the hydantoin racemase is subjected to a mutagenesis,
 b) the nucleic acid sequences obtainable from a) are cloned into a suitable vector and the vector is transferred into a suitable expression system, and

c) the resulting hydantoin racemases having improved activity and/or selectivity and/or stability are detected by means of a process according to one or more of claims 1 to 4 and isolated.

6. rec-Polypeptides or nucleic acid sequences coding therefor obtainable according to claim 5.

7. Use of the polypeptides according to claim 6 in the preparation of enantiomerically enriched N-carbamoyl-amino acid or amino acids.

8. Use of the nucleic acid sequences according to claim 6 in the preparation of whole cell catalysts.

9. Hydantoin racemase containing in position 79 an amino acid substitution with an amino acid selected from the group consisting of A, R, N, D, C, Q, E, H, I, L, K, M, F, P, S, T, Y and V.

10. Hydantoin racemases containing the consensus sequence FX_1DX_2GL (Seq. 1), wherein X_2 represents P or T and X_1 represents in position 79 an amino acid selected from the group A, R, N, D, C, Q, E, H, I, L, K, M, F, P, S, T, Y or V.

11. Isolated nucleic acid sequence coding for a hydantoin racemase selected from the group:

a) a nucleic acid sequence coding for a hydantoin racemase according to claim 9 and/or 10,

b) a nucleic acid sequence which hybridises under stringent conditions with the nucleic acid sequence coding for a hydantoin racemase according to claim 9 and/or 10 or with the sequence complementary thereto,

c) a nucleic acid sequence according to Seq.ID.No. 3, 5, 7 or 9 or a nucleic acid sequence having a homology of > 80% therewith,

d) a nucleic acid sequence containing 15 successive

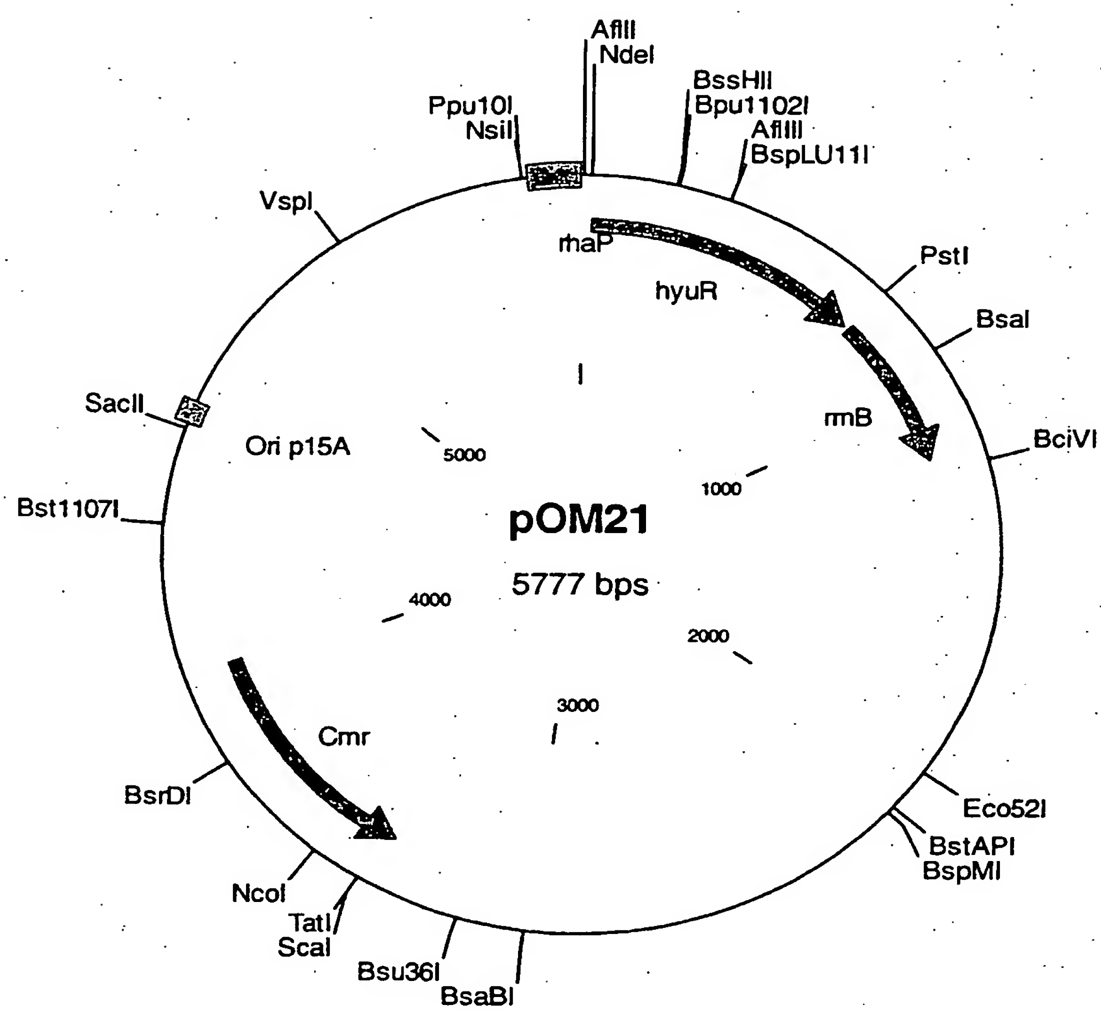
nucleotides of the sequences Seq.ID.No. 3, 5, 7 or 9.

12. Whole cell catalyst containing a cloned gene for a hydantoin racemase according to claims 9 and/or 10.

13. Plasmids, vectors or microorganisms containing a nucleic acid sequence according to claim 9 and/or 10.

14. Primers for the preparation of the nucleic acid sequences according to claim 9 and/or 10 by means of PCR.

Fig. 1: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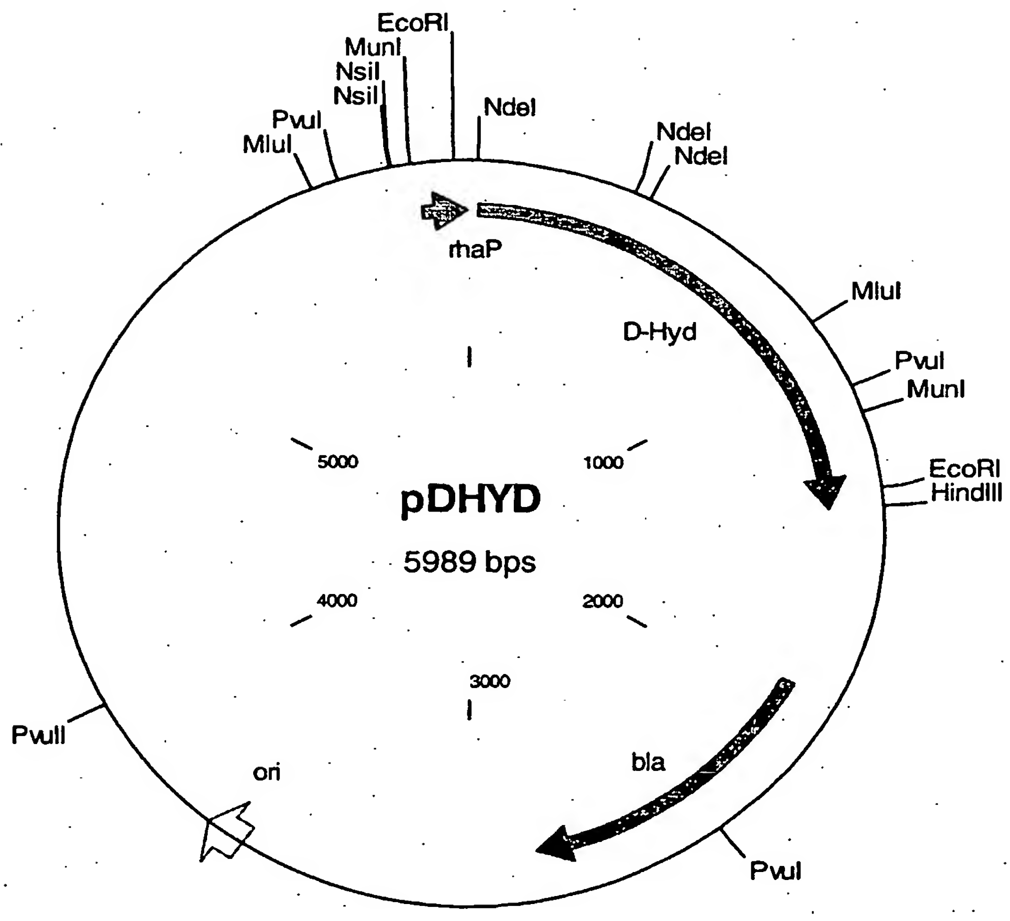
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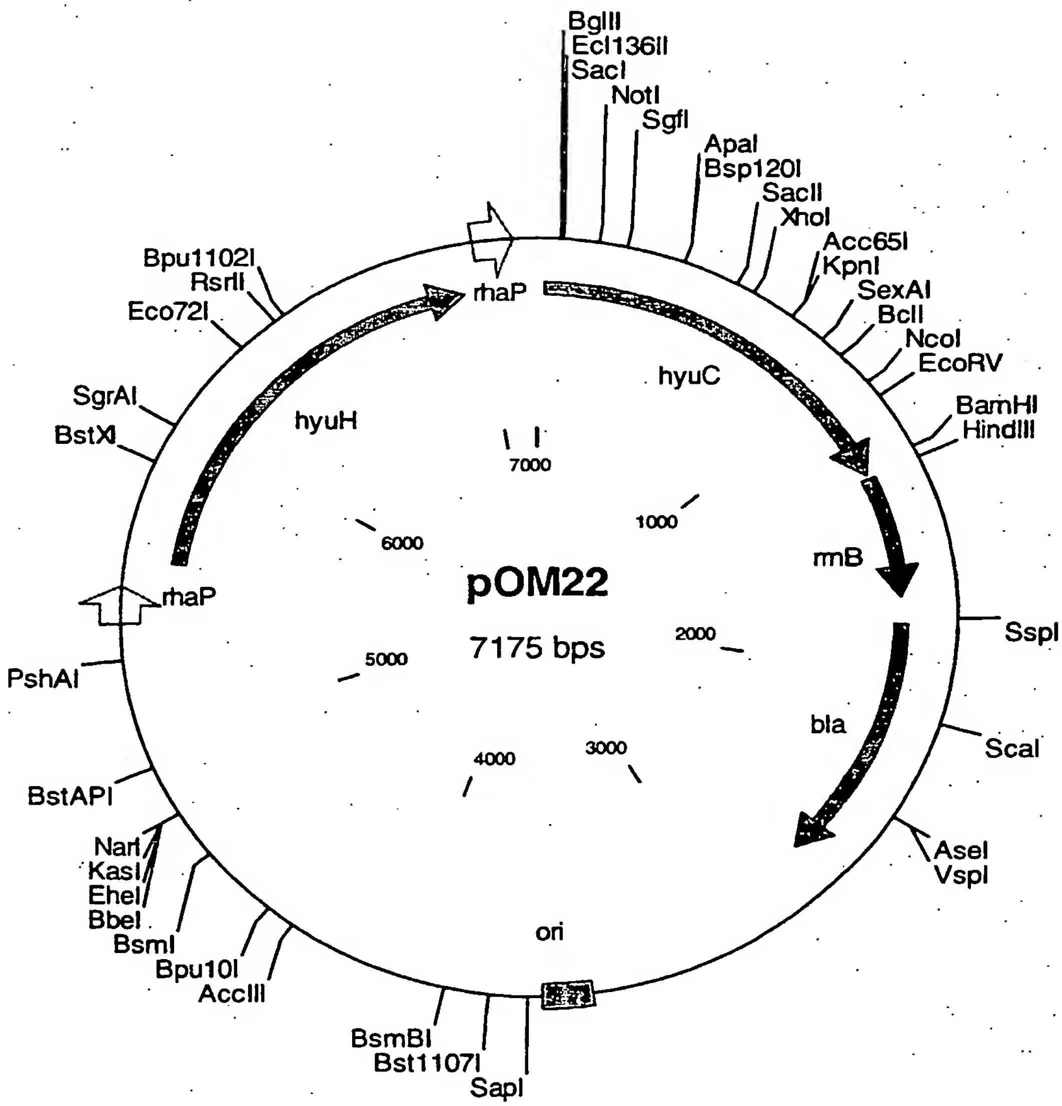
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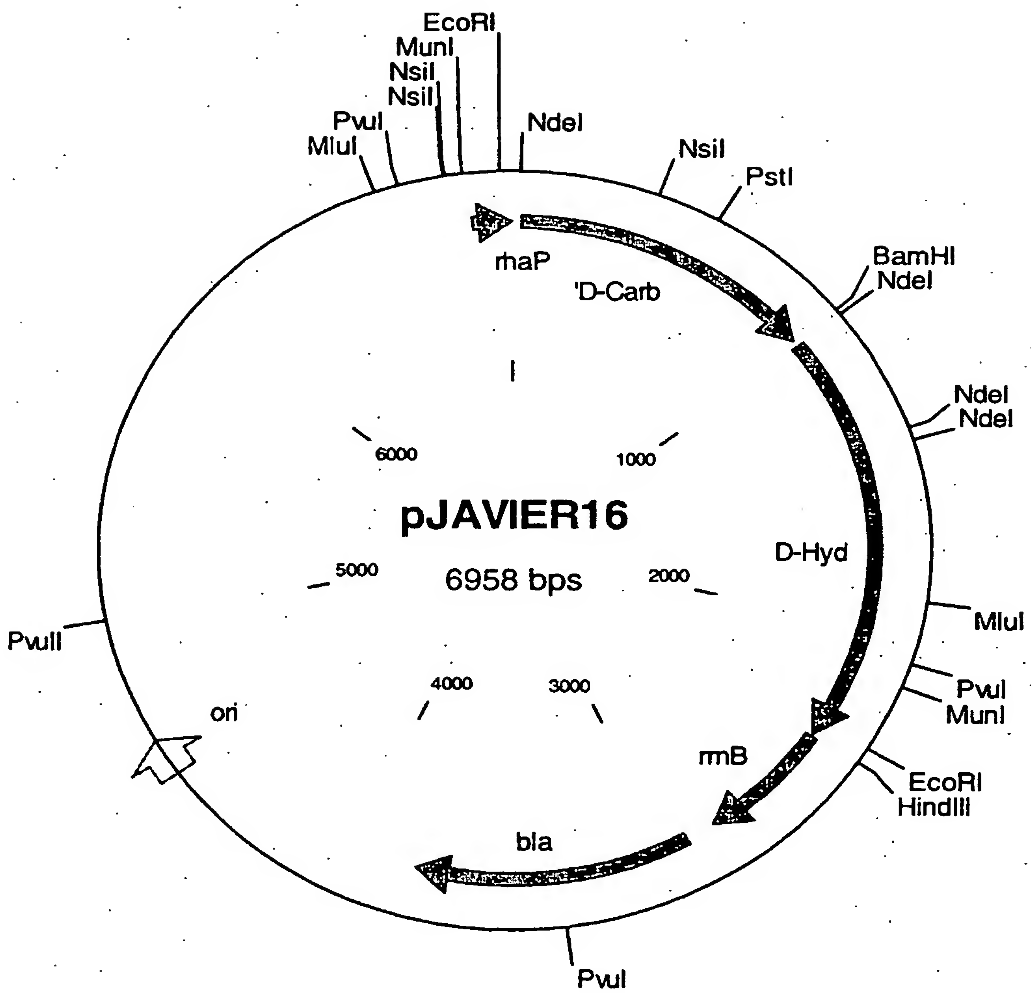
Fig. 4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Ser Ile Val Ser Ile Leu Pro Arg Val Arg Lys His Leu His Glu Leu
        115                 120                 125

gta cgg caa gcg ggg gcg acg aat cgc ctc gcc tcc atc aag ctc cca    432
Val Arg Gln Ala Gly Ala Thr Asn Arg Leu Ala Ser Ile Lys Leu Pro
    130                 135                 140

aat ctg ggg gtg atg gcc ttc cat gag gac gaa cat gcc gca ctg gag    480
Asn Leu Gly Val Met Ala Phe His Glu Asp Glu His Ala Ala Leu Glu
145                 150                 155                 160

acg ctc aaa caa gcc gcc aag gag gcg gtc cag gag gac ggc gcc gag    528
Thr Leu Lys Gln Ala Ala Lys Glu Ala Val Gln Glu Asp Gly Ala Glu
                165                 170                 175

tcg ata gtg ctc gga tgc gcc ggc atg gtg ggg ttt gcg cgt caa ctg    576
Ser Ile Val Leu Gly Cys Ala Gly Met Val Gly Phe Ala Arg Gln Leu
            180                 185                 190

agc gac gaa ctc ggc gtc cct gtc atc gac ccc gtc gag gca gct tgc    624
Ser Asp Glu Leu Gly Val Pro Val Ile Asp Pro Val Glu Ala Ala Cys
        195                 200                 205

cgc gtg gcc gag agt ttg gtc gct ctg ggc tac cag acc agc aaa gcg    672
Arg Val Ala Glu Ser Leu Val Ala Leu Gly Tyr Gln Thr Ser Lys Ala
    210                 215                 220

aac tcg tat caa aaa ccg aca gag aag cag tac ctc tag                711
Asn Ser Tyr Gln Lys Pro Thr Glu Lys Gln Tyr Leu
225                 230                 235
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<210> 8
<211> 237

<212> PRT
<213> Artificial sequence
<223> Description of the artificial sequence:AE3

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Ser Val Ala Asp Ala Ala Gln Gln Val Val Ala Thr Gly Thr Ile Ile
20 25 30

Ser Ala Ile Asn Pro Ser Arg Gly Pro Ala Val Ile Glu Gly Ser Phe
35 40 45

Asp Glu Ala Leu Ala Thr Phe His Leu Ile Glu Glu Val Glu Arg Ala
50 55 60

Glu Arg Glu Asn Pro Pro Asp Ala Tyr Val Ile Ala Cys Phe Gln Asp
65 70 75 80

Pro Gly Leu Asp Ala Val Lys Glu Leu Thr Asp Arg Pro Val Val Gly
85 90 95

Val Ala Glu Ala Ala Ile His Met Ser Ser Phe Val Ala Ala Thr Phe
100 105 110

Ser Ile Val Ser Ile Leu Pro Arg Val Arg Lys His Leu His Glu Leu
115 120 125

Val Arg Gln Ala Gly Ala Thr Asn Arg Leu Ala Ser Ile Lys Leu Pro
130 135 140

Asn Leu Gly Val Met Ala Phe His Glu Asp Glu His Ala Ala Leu Glu
145 150 155 160

Thr Leu Lys Gln Ala Ala Lys Glu Ala Val Gln Glu Asp Gly Ala Glu
165 170 175

Ser Ile Val Leu Gly Cys Ala Gly Met Val Gly Phe Ala Arg Gln Leu
180 185 190

Ser Asp Glu Leu Gly Val Pro Val Ile Asp Pro Val Glu Ala Ala Cys
195 200 205

Arg Val Ala Glu Ser Leu Val Ala Leu Gly Tyr Gln Thr Ser Lys Ala
210 215 220

Asn Ser Tyr Gln Lys Pro Thr Glu Lys Gln Tyr Leu
225 230 235

<210> 9
<211> 711
<212> DNA
<213> Artificial sequence

<220>
<223> Description of the artificial sequence:BB5

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<221> CDS
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Met Arg Ile Leu Val Ile Asn Pro Asn Ser Ser Ser Ala Leu Thr Glu
1               5                   10                  15

tcg gtt gcg gac gca gca caa caa gtt gtc gcg acc ggc acc ata att     96
Ser Val Ala Asp Ala Ala Gln Gln Val Val Ala Thr Gly Thr Ile Ile
            20                  25                  30

tct gcc atc aac ccc tcc aga gga ccc gcc gtc att gaa ggc agc ttt    144
Ser Ala Ile Asn Pro Ser Arg Gly Pro Ala Val Ile Glu Gly Ser Phe
        35                  40                  45

gac gaa gca ctg gcc acg ttc cat ctc att gaa gag gtg gag cgc gct    192
Asp Glu Ala Leu Ala Thr Phe His Leu Ile Glu Glu Val Glu Arg Ala
    50                  55                  60

gag cgg gaa aac ccg ccc gac gcc tac gtc atc gca tgt ttc ttg gat    240
Glu Arg Glu Asn Pro Pro Asp Ala Tyr Val Ile Ala Cys Phe Leu Asp
65                  70                  75                  80

ccg gga ctt gac gcg gtc aag gag ctg act gac agg cca gtg gta gga    288
Pro Gly Leu Asp Ala Val Lys Glu Leu Thr Asp Arg Pro Val Val Gly
                85                  90                  95

gtt gcc gaa gct gca atc cac atg tct tca ttc gtc gcg gcc acc ttc    336
Val Ala Glu Ala Ala Ile His Met Ser Ser Phe Val Ala Ala Thr Phe
            100                 105                 110

tcc att gtc agc atc ctc ccg agg gtc agg aaa cat ctg cac gaa ctg    384
Ser Ile Val Ser Ile Leu Pro Arg Val Arg Lys His Leu His Glu Leu
        115                 120                 125

gta cgg caa gcg ggg gcg acg aat cgc ctc gcc tcc atc aag ctc cca    432
Val Arg Gln Ala Gly Ala Thr Asn Arg Leu Ala Ser Ile Lys Leu Pro
    130                 135                 140

aat ctg ggg gtg atg gcc ttc cat gag gac gaa cat gcc gca ctg gag    480
Asn Leu Gly Val Met Ala Phe His Glu Asp Glu His Ala Ala Leu Glu
145                 150                 155                 160

acg ctc aaa caa gcc gcc aag gag gcg gtc cag gag gac ggc gcc gag    528
Thr Leu Lys Gln Ala Ala Lys Glu Ala Val Gln Glu Asp Gly Ala Glu
                165                 170                 175

tcg ata gtg ctc gga tgc gcc ggc atg gtg ggg ttt gcg cgt caa ctg    576
Ser Ile Val Leu Gly Cys Ala Gly Met Val Gly Phe Ala Arg Gln Leu
            180                 185                 190

agc gac gaa ctc ggc gtc cct gtc atc gac ccc gtc gag gca gct tgc    624
Ser Asp Glu Leu Gly Val Pro Val Ile Asp Pro Val Glu Ala Ala Cys
        195                 200                 205
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cgc gtg gcc gag agt ttg gtc gct ctg ggc tac cag acc agc aaa gcg    672
Arg Val Ala Glu Ser Leu Val Ala Leu Gly Tyr Gln Thr Ser Lys Ala
    210                 215                 220

aac tcg tat caa aaa ccg aca gag aag cag tac ctc tag                711
Asn Ser Tyr Gln Lys Pro Thr Glu Lys Gln Tyr Leu
225                 230                 235


<210> 10
<211> 237
<212> PRT
<213> Artificial sequence
<223> Description of the artificial sequence:BB5

<400> 10
Met Arg Ile Leu Val Ile Asn Pro Asn Ser Ser Ser Ala Leu Thr Glu
 1               5                  10                  15

Ser Val Ala Asp Ala Ala Gln Gln Val Val Ala Thr Gly Thr Ile Ile
            20                  25                  30

Ser Ala Ile Asn Pro Ser Arg Gly Pro Ala Val Ile Glu Gly Ser Phe
        35                  40                  45

Asp Glu Ala Leu Ala Thr Phe His Leu Ile Glu Glu Val Glu Arg Ala
    50                  55                  60

Glu Arg Glu Asn Pro Pro Asp Ala Tyr Val Ile Ala Cys Phe Leu Asp
65                  70                  75                  80

Pro Gly Leu Asp Ala Val Lys Glu Leu Thr Asp Arg Pro Val Val Gly
                85                  90                  95

Val Ala Glu Ala Ala Ile His Met Ser Ser Phe Val Ala Ala Thr Phe
            100                 105                 110

Ser Ile Val Ser Ile Leu Pro Arg Val Arg Lys His Leu His Glu Leu
        115                 120                 125

Val Arg Gln Ala Gly Ala Thr Asn Arg Leu Ala Ser Ile Lys Leu Pro
    130                 135                 140

Asn Leu Gly Val Met Ala Phe His Glu Asp Glu His Ala Ala Leu Glu
145                 150                 155                 160

Thr Leu Lys Gln Ala Ala Lys Glu Ala Val Gln Glu Asp Gly Ala Glu
                165                 170                 175

Ser Ile Val Leu Gly Cys Ala Gly Met Val Gly Phe Ala Arg Gln Leu
            180                 185                 190

Ser Asp Glu Leu Gly Val Pro Val Ile Asp Pro Val Glu Ala Ala Cys
        195                 200                 205

Arg Val Ala Glu Ser Leu Val Ala Leu Gly Tyr Gln Thr Ser Lys Ala
    210                 215                 220
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Asn Ser Tyr Gln Lys Pro Thr Glu Lys Gln Tyr Leu
225 230 235

<210> 11
<211> 25
<212> DNA
<213> Artificial sequence

<220>
<223> Description of the artifical sequence: Primer5

<400> 11
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<210> 12
<211> 30
<212> DNA
<213> Artificial sequence

<220>
<223> Description of the artificial sequence: Primer6

<400> 12
ggtcaggtgg gtccaccgcg ctactgccgc 30

<210> 13
<211> 5777
<212> DNA
<213> Artificial sequence

<220>
<223> Description of the artificial sequence:Plasmid pOM21

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<211> 7175
<212> DNA
<213> Artificial sequence

<220>
<223> Description of the artificial sequence:Plasmid pOM22

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<213> Artificial sequence

<220>
<223> Description of the artificial sequence:Plasmid pDHYH

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